

Short communication

Loss of cytochrome *c* Fe(III)/Fe(II) redox couple in ionic liquids

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Abstract

The electrochemical behavior of cytochrome *c* (cyt-*c*) adsorbed to gold electrodes through mercaptoalkanoic acid/mercaptoalcohol monolayers was investigated in the 1-butyl-3-methylimidazolium salts of bis(trifluoromethylsulfonyl)imide and hexafluorophosphate. Cyt-*c* does not retain its Fe(III)/Fe(II) redox activity in dry ionic liquids (ILs); however, the redox signal of the modified electrodes can be reconstituted upon subsequent treatment with aqueous buffer. Water saturated ILs, 1.4 wt.%, failed to support the redox activity of the adsorbed cyt-*c*; therefore, the ILs' deleterious effects on the redox behavior of the cyt-*c* modified electrodes cannot be attributed solely to the stripping of essential water from the enzyme's polypeptide lattice.

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1. Introduction

Ionic liquids (ILs) have emerged as a novel non-aqueous, polar class of solvents that hold great promise in the development of green chemical applications and processes [1]. Of considerable interest are recent studies that have shown that ILs are suitable media for supporting biocatalytic processes [2,3]. The enzymes detailed in these studies, however, have typically been limited to hydrolases. Relatively little data exists on the activity of oxidoreductases (e.g. desaturases, oxidases, and peroxidases) in ILs and no data exist on the activity of redox enzyme-modified electrodes in ILs. The conductive nature of ILs [4] make them especially attractive non-aqueous media in which to study redox active enzymes. It is presumable that ILs could support biocatalytic transformations via electrolysis of substrates using enzyme-modified electrodes. Initial investigations proved that the Fe(III)/Fe(II) redox couple of

protoporphyrin(IX) iron(III) chloride (hemin) and hemin-modified electrodes was stable in [alkylimidazolium][PF₆] salts without the need for additional electrolyte [5]. Herein we expand on these findings to report the first study of the redox behavior of a heme-containing protein, cytochrome *c* (cyt-*c*), in 1-butyl-3-methylimidazolium salts of bis(trifluoromethylsulfonyl)imide ([bmim][tf₂N]) and hexafluorophosphate ([bmim][PF₆]).

2. Experimental

2.1. Materials

Horse heart cyt-*c* (cyt-*c*, 95%, stored at −4 °C), 11-mercaptopundecanoic acid (11-MUA), 11-mercaptop-1-undecanol (11-MU), 1-[3-(dimethylamino)propyl]3-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), potassium hydrogen phosphate, potassium dihydrogen phosphate, and anhydrous pyridine (in a Sure/Seal™ bottle) were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. Ethanol (Sigma-Aldrich) was dried over activated 3 Å molecular sieves. Deionized water was purified by filtration through a Barnstead/Thermolyne Corp. (Dubuque, IA) 1056 Series Easy Pure UV/UF Compact

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¹ Product names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may be suitable.

Reagent Grade water system. The ILs, [bmim][tf₂N] and [bmim][PF₆] were prepared as described previously [6,7].

2.2. Electrode preparation

Gold wire electrodes (23 cm × 0.05 cm dia., Bioanalytical Systems) were cleaned with a 'piranha' solution (a 3:1 mixture of conc. H₂SO₄ + 30 wt.% aqueous H₂O₂) followed by sonication in water, repeated three times. The electrodes were then soaked in conc. extracted H₂SO₄ overnight, rinsed with water, treated with HNO₃ for 10 min, again rinsed with water, rinsed with methanol, and used immediately.

Electrode preparations were performed under dinitrogen using standard Schlenk line techniques. Phosphate buffer (2.3 mM K₂HPO₄ + 2.0 mM KH₂PO₄ titrated to pH 7 with 5 M KOH) was degassed under high vacuum for 2 h and sparged with dinitrogen for 2 h. A self-assembled monolayer (SAM) was adsorbed to freshly cleaned gold wire electrodes by immediately rinsing the electrode with phosphate buffer followed by ethanol and incubating in 2 ml equimolar (5 mM) solutions of 11-MUA and 11-MU in ethanol for 24 h. The electrodes were then rinsed with copious amounts of phosphate buffer. Electrostatically held cyt-*c* was formed by incubation of the SAM-modified electrode in a 52 μM aqueous solution (pH 7) of cyt-*c*. The electrodes were either wicked dry or rigorously dried by rinsing with ethanol and standing in a dinitrogen stream for 24 h.

Cyt-*c* was covalently bound to the gold wire electrodes using a similar procedure. A monolayer of 11-MU and 11-MUA was deposited on freshly cleaned gold wire electrodes as described above. The 11-MU:11-MUA monolayer was activated by rinsing the electrode with phosphate buffer and incubating the electrode in a 2 ml aqueous solution of EDC (200 mM) and NHS (50 mM) for 10 min. The electrodes were rinsed with phosphate buffer and incubated in cyt-*c* solutions as described above. The electrodes were then treated with 1.0-M phosphate buffer to remove any electrostatically adsorbed cyt-*c* and rinsed with the water before use.

2.3. Cyclic voltammetry

The electrochemical experiments were performed using a BAS CV-50 W voltammetric analyzer, a BAS C3 cell stand, Au coiled wire electrodes (average electrochemically active area 2.80 ± 0.08 cm²), an aqueous Ag/AgCl/3 M NaCl reference electrode, Pt wire auxiliary electrode, and a low volume, jacketed cyclic voltammetry (CV) cell. The entire system was obtained from Bioanalytical Systems (Lafayette, IN). The water-jacketed cell was heated/cooled using an Isotemp 1016 D circulating bath (Fisher Scientific, Pittsburgh, PA) filled with silicon oil. All electrochemical experiments were

performed under a dry dinitrogen atmosphere at 25 °C (unless otherwise noted) in either 5-ml of phosphate buffer or 5 ml of ILs.

3. Results and discussion

In the present study, cyt-*c* was adsorbed to gold electrodes and used as a model to investigate the performance of membrane-associated redox proteins in ILs. Cyt-*c* is catalytically active in IL solutions using hydrogen peroxide and guaiacol as a substrate [8]. However, cyt-*c* is insoluble in the ILs and can be studied only in the presence of solubilising agents [8]. Immobilising the cyt-*c* on electrodes eliminates the use of these solubilising agents and allows for the most direct study of the redox properties of native cyt-*c* in the ILs. Cyt-*c* was adsorbed to freshly cleaned gold wire electrodes through SAMs formed from equimolar amounts of 11-MUA and 11-MU. The use of mixed SAMs of mercaptoalkanoic acids and mercaptoalcohols has been shown to result in superior electron transfer rates of immobilized cyt-*c* in aqueous buffers [9,10]. The immobilisation of cyt-*c* to the SAM on the gold surface was either through an electrostatic or covalent interaction.

The CVs of cyt-*c* electrostatically adsorbed to a gold electrodes through a 11-MUA:11-MU SAM (cyt-*c*/11-MU:11-MUA/Au) were recorded in 10 mM ionic strength phosphate buffer to establish the veracity of our methods. A reversible Fe(III)/Fe(II) redox couple was observed for the cyt-*c* modified electrodes with a formal potential (E°) of -30 ± 9 mV vs. Ag/AgCl ($n = 6$ electrodes) and a peak separation (ΔE) of 19 ± 10 mV (Table 1). These results are in agreement with values reported in the literature for cyt-*c* adsorbed to gold through 11-MUA [11,12] and mixed 11-MUA:11-MU [10] SAMs. Control experiments measuring the CV of 11-MUA:11-MU/Au electrodes in phosphate buffer showed that the carboxylic acid-terminated SAMs were electrochemically inactive. This is contrary to reports that show electrochemical responses of carboxylic acid groups in phosphate buffer when functionalized on single-wall carbon nanotubes that were adsorbed to glassy carbon electrodes [13]. The surface coverage of redox-active cyt-*c* ($\Gamma^{\circ}_{\text{cyt-c}}$) was found to be 4.0 ± 0.8 pmol cm⁻² ($n = 6$ electrodes) based on the cathodic peak currents obtained by CV. Our $\Gamma^{\circ}_{\text{cyt-c}}$ values are approximately half those reported in the literature [10]. Covalent attachment of cyt-*c* to the SAM did not appreciably alter the E° , ΔE , and $\Gamma^{\circ}_{\text{cyt-c}}$ values (Table 1). The integrity of the redox signal obtained with both the electrostatically and covalently cyt-*c*-modified electrodes was maintained in the phosphate buffer over 10 consecutive scans and upon standing in buffer for 2 h.

Table 1
Electrochemical data for Cyt-*c* electrostatically adsorbed and covalently bound to gold electrodes

Solvent	$E^{\circ'} \pm \sigma/\text{mV}$	$\Delta E \pm \sigma/\text{mV}$	$I_c \pm \sigma/\mu\text{A}$	I_c/I_a	$\Gamma^{\circ} \pm \sigma/\text{pmol cm}^{-2}$
Phosphate buffer ^{a,b}	-30 ± 9	19 ± 10	0.50 ± 0.12	0.88 ± 0.07	4.0 ± 0.8
[bmim][tf ₂ N] ^{a,c}	20 ± 7	11 ± 12	0.45 ± 0.12	0.84 ± 0.10	–
[bmim][PF ₆] ^{a,c}	18 ± 6	13 ± 8	0.32 ± 0.26	0.92 ± 0.06	–
Phosphate buffer ^{d,b}	-23 ± 6	23 ± 6	0.57 ± 0.12	0.93 ± 0.04	3.5 ± 0.4
[bmim][tf ₂ N] ^{d,c}	15 ± 11	26 ± 12	0.39 ± 0.15	1.1 ± 0.10	–
[bmim][PF ₆] ^{d,c}	24 ± 5	15 ± 7	0.35 ± 0.14	1.1 ± 0.12	–

Data were obtained at 50 mV s^{-1} , 25°C , vs. Ag/AgCl, were background subtracted, and are reported with relative S.D.s ($n \geq 3$ electrodes).

^a Electrodes modified with cyt-*c* electrostatically adsorbed to the gold electrodes.

^b Buffer consisted of aqueous $2.3 \text{ mM K}_2\text{HPO}_4$ and $2.0 \text{ mM KH}_2\text{PO}_4$ titrated to pH 7.

^c Data obtained after wicking away the aqueous buffer and within 1 min of immersing the electrodes in the dried ILs (7 ppm water).

^d Electrodes modified with cyt-*c* covalently bound to gold electrodes.

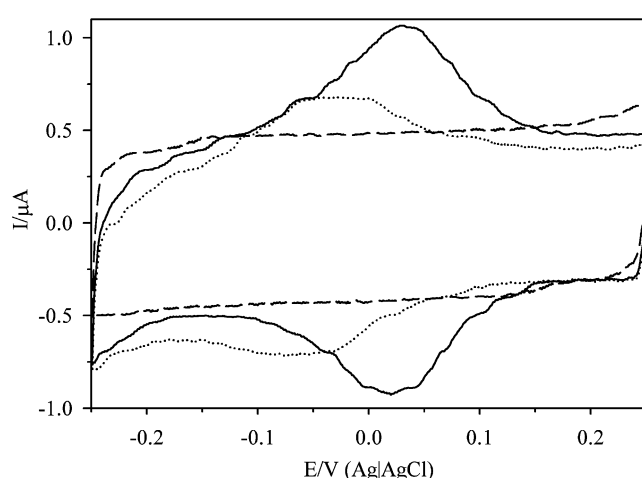


Fig. 1. CV of a cyt-*c*/11-MU:11-MUA/Au electrode in (—) [bmim][tf₂N] after 1 min (---) [bmim][tf₂N] after 1 h, and (···) phosphate buffer for 5 min. CV recorded at 25°C , 50 mV s^{-1} , vs. Ag/AgCl.

Fig. 1 shows the CV of an electrostatically bound cyt-*c*/11-MU:11-MUA/Au electrode in dry [bmim][tf₂N] (7 ppm water determined by Karl Fischer coulometry). The redox couple of the electrode was first established in phosphate buffer to verify the typical cyt-*c* redox signal then the electrode was dried by wicking away the buffer. The electrode was transferred to the dry IL where a reversible Fe(III)/Fe(II) redox couple was observed by CV. The formal potential of the cyt-*c* was shifted approximately 50 mV more positive in the IL compared to phosphate buffer (Table 1). It should be noted that the ILs solutions do not share a common ion with the aqueous reference electrode. Thus an undetermined junction potential exists in the circuit. Therefore, the formal potentials of the immobilized cyt-*c* obtained in the ILs and in phosphate buffer, while similar, cannot be compared directly. The ΔE in ILs was unchanged while the intensity of the cathodic peak current was slightly lower. The cyt-*c* redox signal was stable over 10 consecutive cycles.

Prolonged exposure of the electrodes to [bmim][tf₂N] or [bmim][PF₆] resulted in the loss of the Fe(III)/Fe(II) redox signal. The electrodes, while stable over 10 consecutive cycles measured within the first 5 min of exposure to the IL, become electrochemically inactive after 1-h incubation in the IL (Fig. 1). Electrodes modified with only a 11-MU:11-MUA monolayer were incubated in [bmim][tf₂N] for 24 h, rinsed with ethanol, and then contacted with aqueous cyt-*c* following the established protocol. CVs of these electrodes recorded in phosphate buffer and in IL produced the characteristic redox signals, thus eliminating the possibility that the IL disrupts the 11-MU:11-MUA monolayer or its ability to bind cyt-*c*. Therefore, these findings suggest that the loss of the redox signal is the consequence of the IL deleteriously affecting the adsorbed cyt-*c*.

The loss of the cyt-*c*/11-MU:11-MUA/Au electrode redox signal cannot be attributed to the desorption of cyt-*c* from the 11-MU:11-MUA monolayer. The redox signal that was lost due to exposing the electrode to the IL for 1 h was reconstituted by rinsing the electrode and incubating in phosphate buffer for 5 min, although the reconstituted redox signal was typically less intense (Fig. 1c). Additionally, cyt-*c* can be covalently bound to the electrode by activating the 11-MU:11-MUA monolayer with EDC and NHS followed by treatment with cyt-*c* [10]. The covalently modified cyt-*c*/11-MU:11-MUA/Au electrodes generated a redox signal in the IL that was indistinguishable from that obtained with the electrostatically modified electrodes. The covalently modified electrodes also became electrochemically inactive when exposed to IL for 1 h, and the redox signal was reconstituted upon treatment with phosphate buffer. These results prove that the loss of the electrochemical activity of the Fe(III)/Fe(II) redox couple is not the result of cyt-*c* desorption from the electrode. The loss of the cyt-*c* redox activity is most likely to be due to denaturing of the protein caused by contact with the IL possibly resulting in a detrimental reorientation of the heme group away from the electrode surface [14]. Close

alignment of the heme crevice with the electrode surface is essential for heterogeneous electron transfer.

Reorientation of cyt-*c* and the loss of the electrochemical activity of the cyt-*c*/11-MU:11-MUA/Au electrodes upon exposure to the IL may be due to the removal of water from the protein. Polar organic solvents generally result in the loss of enzyme function [15]. This is typically attributed to the solvent's propensity to strip essential water from the enzyme generating a protein conformation that is less active or inactive [16]. For example, the electron transfer rate of cyt-*c* has been shown to decrease as the organic solvent content of the media increases [17]. The ILs used in this study have been shown to be hygroscopic [18]. Therefore, the ILs are presumably as capable of stripping essential water from the enzyme as some polar organic solvents. Cyt-*c*/11-MU:11-MUA/Au electrodes that were thoroughly dried under a flow of nitrogen for 24 h after removal from the phosphate buffer were not electrochemically active in the IL. The same electrodes, however, did produce a redox signal upon rinsing and reimmersion in the phosphate buffer. Cyt-*c* modified electrodes that were simply wicked dry, and thus most likely still partially hydrated, displayed redox activity in the IL which suggests that a hydration layer is necessary to protect the bound cyt-*c* from the IL and is essential in preserving the redox activity of the electrodes. However, if the water activity of the IL was the lone determinate in the preservation of the redox activity of the bound cyt-*c*, water saturated [bmim][tf₂N], (1.4 wt.% determined by Karl Fischer coulometry) should support redox activity. This was not the case. Therefore, other confounding effects must contribute to the IL's deleterious effects on the redox activity of the bound cyt-*c*.

4. Conclusions

The results presented herein show that immobilised cyt-*c* was not electrochemically active after prolonged exposure to ILs. Free cyt-*c* modified with solubilising agents, however, remains catalytically active in ILs [8]. This suggests that cyt-*c* immobilised on gold electrodes

could remain electrochemically and catalytically active if insulated from the IL. Efforts are ongoing to assemble cyt-*c* modified electrodes with the enzyme encased in a protective layer (e.g. a sol–gel layer or lipid bilayer) that will permit electron transfer and interaction with a substrate in ILs.

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